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# Transcription factors in alkaloid biosynthesis

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## Abstract

Higher plants produce a large variety of low-molecular-weight secondary compounds. Among them, nitrogen-containing alkaloids are the most biologically active, and are often used pharmaceutically. Whereas alkaloid chemistry has been intensively investigated, alkaloid biosynthesis, including the relevant biosynthetic enzymes, genes and their regulation, and especially transcription factors, are largely unknown, since only a limited number of plant species produce certain types of alkaloids and they are difficult to study. Recently, however, several groups have succeeded in isolating the transcription factors that are involved in the biosynthesis of several types of alkaloids, including bHLH, ERF, and WRKY. Most of them show Jasmonate (JA)-responsiveness, which suggests that the JA signaling cascade plays an important role in alkaloid biosynthesis. Here, we summarize the types and functions of transcription factors that have been isolated in alkaloid biosynthesis, and characterize their similarities and differences compared to those in other secondary metabolite pathways; i.e., phenylpropanoid, and terpenoid biosynthesis. The evolution of this biosynthetic pathway and regulatory network, as well as the application of these transcription factors to metabolic engineering, are discussed.

## Key Words

Alkaloid biosynthesis, Transcription factor, Jasmonate signaling, Transcriptional regulation, Secondary metabolism

## Abbreviations

ADC; arginine decarboxylase, ADS; amorpha-4, 11-diene synthase, AOX; aspartate oxidase, AP2; APETALA2, AS; anthranilate synthase, BBE; berberine bridge enzyme, bHLH; basic Helix-Loop-Helix, BIAs; benzyloquinoline alkaloids, BPF-1; box P-binding factor-1, BY-2; Bright Yellow-2, bZIP; basic-region leucine zipper, CHI; chalcone isomerase, CHS; chalcone synthase, CM; chorismate mutase, CNMT; coclaurine-*N*-methyltransferase, COI1; Coronatine Insensitive 1, CPR; cytochrome P450 reductase, CYP71AV1; amorpha-4,11-diene monooxygenase, CYP80B1; (*S*)-*N*-methylcoclaurine 3'-hydroxylase, CYP719A1; canadine synthase, CYP719A2/A3; stylophine synthase, CYP719A5; cheilanthifoline synthase, CYP719B1; salutaridine synthase, D4H; desacetoxyvindoline 4-hydroxylase, DAH7PS; 3-deoxy-D-arabino heputulosonate 7-phosphate synthase, DAT; deacetylvindoline-4-*O*-acetyltransferase, DFR; dihydroflavonol reductase, DREB;

Dehydration-responsive element binding protein, ds; double stranded, DQSDH; dehydroquinase shikimate dehydrogenase, DXS; D-1-deoxyxylulose 5-phosphate synthase, EREBPs; ethylene-responsive element binding proteins, ERFs; ethylene-responsive factors, EAR; ERF-associated amphiphilic repression, EGL3; Enhancer of Glabra 3, EMSA; electrophoretic mobility shift assay, ET; ethylene, F3H; flavanone 3-hydroxylase, F3'H; flavonoid 3'-hydroxylase, G10H; geraniol 10-hydroxylase, GA; gibberellic acid, GAPDH; glyceraldehyde-3-phosphate dehydrogenase, GBF; G-box binding factor, GL3; Glabra 3, JA, jasmonate, JAM1; JA-factor stimulating MAPKK1, JAZ; jasmonate ZIM domain, JID; JAZ-interaction domain, JRE; JA-responsive element, MKK3; MAPK kinase 3, MPK6; mitogen-activated protein kinase 6, MATE1/2; nicotine transport, MeJA; methyl-JA, MIAs; monoterpenoid indole alkaloids, MPO; *N*-methylputrescine oxidase, NAC; (NAM/ATAF1,2/CUC2), NCS; norcoclaurine synthase, ODC; ornithine decarboxylase, 4'OMT; 3'-hydroxy-*N*-methylcoclaurine-4'-*O*-methyltransferase, 6OMT; norcoclaurine 6-*O*-methyltransferase, ORA59; Octadecanoid-Responsive Arabidopsis AP2/ERF 59, ORCA; Octadecanoid derivative responsive Catharanthus AP2-domain, PAP1; Production of Anthocyanin Pigment 1, PDF1.2; Plant Defensin 1.2, PMT; putrescine *N*-methyltransferase, QPT; quinolinate phosphoribosyltransferase, QS; quinolinic acid synthase, RGA; repressor of GA1-3, RGL3; RGA-LIKE3, RNAi; RNA interference, SPDS; spermidine synthase, STR; strictosidine synthase, TPI; trypsin proteinase inhibitor, TDC; tryptophan decarboxylase, TPL; TOPLESS, TPS; terpene synthase, TT8; Transparent Testa 8, TYDC; tyrosine decarboxylase, UF3GT; UDP-3-*O*-glucosyltransferases, VIGS; Virus-induced gene silencing, VSP; Vegetative Storage Protein, WT; wild type, ZCT; zinc finger Catharanthus transcription factor.



## 1. Introduction

Higher plants produce structurally divergent low-molecular-weight chemicals such as phenylpropanoids, terpenoids, and alkaloids. These secondary metabolites function in defense against pathogens and herbivores, and also attract pollinators (Pichersky and Gang, 2000). These chemicals are also used as essential oils, flavorings, dyes, and pharmaceuticals. In particular, alkaloids, which are nitrogen-containing compounds that are found in about 20% of plant species, have potent biological activities and are used as pharmaceuticals, stimulants, narcotics and poisons (Facchini, 2001).

One of the unique features of alkaloids is their plant species-specific localization. For example, nicotine and tropane alkaloids are mainly found in Solanaceae plants (Hashimoto and Yamada, 1994); nicotine alkaloids are found in *Nicotiana* species and are used in the cigarette industry. The tropane alkaloids hyoscyamine and scopolamine, which are acetylcholine antagonists, are found in *Hyoscyamus*, *Duboisia*, *Atropa*, and *Scopolia* species. Monoterpenoid indole alkaloids (MIAs), which comprise one of the largest groups of plant secondary metabolites and include the antineoplastic agents vinblastine and vincristine obtained from *Catharanthus roseus* (Apocynaceae), occur mainly in the Apocynaceae, Loganiaceae and Rubiaceae (Facchini and De Luca, 2008). Benzyloquinoline alkaloids (BIAs), another large group of natural products that include approximately 2500 compounds, are found mainly in the Papaveraceae, Ranunculaceae, Berberidaceae and Menispermaceae (Kutchan, 1995). Especially, the analgesics morphine and codeine, are exclusively found in *Papaver somniferum* (Papaveraceae), whereas the antimicrobial agents berberine and sanguinarine are found in a rather wide range of plant species; e.g., berberine in *Coptis japonica* (Ranunculaceae) and *Berberis vulgaris* (Berberidaceae), and sanguinarine in *Eschscholzia californica* (Papaveraceae) and *Sanguinaria canadensis* (Papaveraceae).

Research on plant alkaloid chemistry began with the isolation of morphine in 1806. After a long, difficult period of biochemical studies to characterize the biosynthetic pathway using intact plants, cell cultures that produce high levels of secondary metabolites were established, and these offer clear advantages for elucidating alkaloid biosynthesis at the enzyme level (Zenk, 1991, Sato, 2013). Further progress in molecular biological techniques in the late 1980s facilitated the isolation of considerable numbers of genes involved in the biosynthesis of nicotine and tropane alkaloids, MIAs, and BIAs (Sato et al., 2007). However, the mechanism of transcriptional regulation in the biosynthesis of each alkaloid is still unknown.

Recently, transcriptional regulators involved in nicotine, MIA, and BIA

biosynthesis have been reported. In this review, we summarize the types of transcription factors involved in nicotine, MIA, and BIA biosynthesis, and regulation through the jasmonate signaling cascade. We also characterize the similarities and differences between the regulatory mechanisms in alkaloid biosynthesis in comparison to those in phenylpropanoid and terpenoid biosynthesis. Finally, research on transcription factors in alkaloid biosynthesis is discussed from the perspective of industrial production.

## 1. 1 General introduction to transcription factors in plants

Transcription factors regulate gene expression through specific binding to *cis*-acting elements in the promoters of the target genes. Whereas higher plants often produce secondary metabolites in response to environmental abiotic and biotic stresses, these signals, including pathogen-derived molecules called elicitors, trigger the expression of some transcription factor genes through the production of jasmonate, a plant hormone, to induce defense-related genes to produce defense proteins and secondary metabolites, including alkaloids (Table 1) (Zhao et al., 2005; Pauwels et al., 2009). Several transcription factors that participate in the defense responses of plants have been well-characterized.

The AP2/ERF family, a large group of plant-specific transcription factors (Gutterson and Reuber, 2004), have AP2/ERF-type DNA binding domains of approximately 60 amino acids. The AP2/ERF domain was first found in the *Arabidopsis* homeotic gene, *APETALA2* (*AP2*), and also in tobacco ethylene-responsive element binding proteins (EREBPs; later renamed ethylene-responsive factors, ERFs) (Jofuku et al., 1994; Ohme-Takagi and Shinshi, 1995). The AP2/ERF family is divided into four subfamilies: AP2, RAV, ERF, and DREB. Whereas the ERF and DREB subfamilies have only one AP2/ERF domain, the AP2 subfamily has double AP2/ERF domains and the RAV subfamily has one AP2/ERF domain and an additional B3 DNA binding domain. Proteins in the DREB and ERF subfamilies are known to be involved in dehydration or ethylene responses through binding to a dehydration responsive element (DRE) sequence (A/GCCGAC) and an ethylene responsive element (ERE) sequence (AGCCGCC), respectively (Mizoi et al., 2012).

The WRKY family is also found only in plants. After the first isolation of a WRKY protein, SPF1 from sweet potato in 1994 (Ishiguro and Nakamura, 1994), WRKYs have been shown to play roles in defense-response, development, and senescence (Eulgem et al., 2000; Eulgem and Somssich, 2007; Rushton et al., 2010). They all contain the 60-amino acid WRKY domain, which contains a highly conserved

amino acid sequence WRKYGQK at the N-terminal end and a zinc-finger-like motif at the C-terminal end, from which its name is derived. The WRKY family can be divided into three groups based on their structures. Group I has two WRKY domains, whereas Groups II and III have a single domain. Groups I and II have a zinc-finger structure of C2H2, whereas Group III has C2HC. Group II WRKY can be further divided into five subgroups (IIa, IIb, IIc, IId, and IIE) based on the primary amino acid sequence. The WRKY domain specifically recognizes the W-box DNA sequence motif (TTGACC/T) (Rushton et al., 2010).

Proteins of the Basic Helix-Loop-Helix (bHLH) family are not plant-specific, but rather are broadly distributed in eukaryotic organisms where they are involved in many essential biological processes. In animals, bHLH transcription factors perceive environmental signals and regulate the cell cycle, the circadian rhythm, and diverse developmental processes (Atchley and Fitch, 1997; Stevens et al., 2008). Plant bHLH transcription factors have also been shown to be involved in a wide and diverse array of physiological and developmental processes including light signaling, stress response, fruit and flower development, and root development, after a plant bHLH from *Zea mays* was first shown to be involved in flavonoid/anthocyanin biosynthesis (Ludwig et al., 1989; Buck and Atchley, 2003; Li et al., 2006; Carretero-Paulet et al., 2010). The bHLH domain is composed of approximately 60 amino acids, with two functionally different regions. The N-terminal end of the bHLH domain of 15-20 amino acids is involved in DNA-binding and contains six basic residues, whereas the HLH region contains two amphipathic alpha helices with a linking loop of variable lengths and sequences. The amphipathic alpha helices composed of two bHLH proteins promote protein-protein interaction, which allows the formation of homodimeric or heterodimeric complexes. The bHLH proteins have been shown to recognize the so-called core E-box hexanucleotide consensus sequence (CANNTG) through certain conserved amino acids in the basic region, whereas other residues would provide specificity for a given type of E-box (e.g., the G-box; CACGTG). Based on the DNA-binding domain sequences and functional properties, bHLH proteins have been divided into six main groups (Groups A to F) in animals (Atchley and Fitch, 1997). Group A can bind to the E-box sequence, whereas Group B specifically binds to the G-box sequence. Group C contains an additional protein-protein interaction PAS domain and can bind to an E-box-like sequence. Group D lacks the basic region and forms heterodimers with typical bHLH proteins. Group E contains a conserved Pro or Gly residue in the basic region, and preferentially binds to CACGNG. Group F includes COE-bHLH proteins and has divergent sequences compared to the other groups. Most plant bHLH proteins belong to

Group B.

## 2. Transcription factors in alkaloid biosynthesis

### 2.1 AP2/ERF-domain transcription factors

The first transcription factors in alkaloid biosynthesis to be isolated were AP2/ERF-domain type octadecanoid jasmonate (JA)-responsive ORCA1 and ORCA2 in MIA biosynthesis in *Catharanthus roseus* (Fig. 1). They were isolated using JA-responsive regions in *Strictosidine synthase* (*STR*) promoter, which contain GCC-box-like elements, through the use of yeast one-hybrid screening (Menke et al., 1999a).

ORCA1 and ORCA2 belong to different subfamilies, i.e., ORCA1 is homologous to DREB2A and DREB2B in drought-responsive gene expression (Sakuma et al., 2002), whereas ORCA2 is highly homologous to tobacco EREBP1 and tomato Pti4 in defense-responsive gene expression (Ohme-Takagi and Shinshi, 1995; Gu et al., 2000). ORCA1 could bind to the JA-responsive element of *STR* promoter *in vitro* but had little transcriptional activity *in vivo*, and treatment with methyl-JA (MeJA) did not induce the expression of the *ORCA1* gene. On the other hand, ORCA2 not only binds to the JA-responsive element, but also showed *in vivo* transactivation activity and JA-responsive expression. Thus, Menke et al. (1999a) concluded that ORCA2 acts as a transcriptional activator of *STR* promoter.

>>Figure 1

Later, van der Fits and Memelink (2000) isolated another AP2/ERF-domain protein (named ORCA3 based on its similarity to ORCA2) from *C. roseus* using the activation tagging approach. Overexpression of the *ORCA3* gene induced several MIA biosynthetic genes (e.g., *STR*, *tryptophan decarboxylase* (*TDC*), *cytochrome P450 reductase* (*CPR*), and *desacetoxylvindoline 4-hydroxylase* (*D4H*)), and also some primary metabolism genes involved in the biosynthesis of precursors of MIA (i.e., *anthranilate synthase* (*AS*) and *D-1-deoxyxylulose 5-phosphate synthase* (*DXS*)), whereas the expression of genes for several enzymes in MIA biosynthesis, i.e., *geraniol 10-hydroxylase* (*G10H*) and *deacetylvindoline-4-O-acetyltransferase* (*DAT*), and many genes involved in primary metabolism were not affected (van der Fits and Memelink, 2000). The accumulation of tryptophan and tryptamine in cells that overexpressed *ORCA3* and an increase in MIA production under the extracellular feeding of a terpenoid precursor, loganin, indicate that ORCA3 is a master regulator, but is not

sufficient for MIA biosynthesis. Since treatment with JA induces MIA biosynthesis and both the *ORCA2* and *ORCA3* genes have been shown to respond to JA, *ORCA2* and *ORCA3* might regulate MIA biosynthesis in cooperation with other transcription factors in MIA biosynthesis, as discussed below (van der Fits and Memelink, 2001; Memelink et al., 2001, and also see below).

AP2/ERF-domain transcription factors have also been isolated from tobacco, which produces a variety of secondary metabolites, e.g., nicotine, anabasine, and anatabine, in a JA-responsive fashion (Imanishi et al., 1998)(see nicotine biosynthetic pathway in Fig. 2). Using cDNA-amplified fragment length polymorphism (AFLP) analysis in JA-treated cultured tobacco Bright Yellow-2 (BY-2) cells in combination with a target metabolite analysis, Goossens et al. (2003) found two AP2/ERF-domain proteins and nicotine biosynthetic enzyme genes among approximately 600 MeJA-modulated transcript tags. The transactivation activities of these AP2/ERF-type transcription factors, named *NtORC1/ERF221* and *NtJAP1/ERF10*, were confirmed by the measurement of *putrescine N-methyltransferase (PMT)* gene expression using a transient luciferase-reporter assay in BY-2 protoplasts (De Sutter et al., 2005; Rushton et al., 2008).

The further investigation of a stable tobacco transformant that overexpressed *NtORC1/ERF221*, a homologue of *ORCA3*, showed a high productivity of nicotine and other pyridine alkaloids without MeJA treatment, whereas similar results were not seen with the overexpression of *NtJAP1/ERF10* (De Boer et al., 2011), which indicates that *NtORC1/ERF221* plays a key role in nicotine alkaloid biosynthesis. Interestingly, the maximum activity of *NtORC1/ERF221* requires both GCC-box and G-box, since the transcriptional activity of *NtORC1/ERF221* was markedly reduced in a transient reporter assay using mutant *PMT* promoter that contained not only mutant GCC-box, but also mutant G-box, a target of bHLH transcription factors (De Boer et al., 2011). A further analysis of the co-expression of *NtORC1/ERF221* and *NbbHLH1*, a positive regulator of nicotine biosynthesis isolated from *Nicotiana benthamiana*, supports that they have synergistic effects on the expression of *PMT* and *quinolinate phosphoribosyltransferase (QPT)*(De Boer et al., 2011).

>> Figure 2

AP2/ERF domain transcription factor genes were also independently isolated using a microarray analysis of a tobacco *nic* mutant (Shoji et al., 2010). *nic* mutant, which lacks two distinct regulatory loci called *NIC1* and *NIC2*, has low levels of

nicotine and is used to isolate enzyme and transporter genes involved in nicotine biosynthesis (Hibi et al., 1994; Katoh et al., 2007; Shoji et al., 2009). In *nic* mutant, the expression of several genes involved in the biosynthesis of nicotine and its precursors (i.e., *PMT*, *A622*, *ornithine decarboxylase (ODC)*, *aspartate oxidase (AOX)*, *quinolinic acid synthase (QS)*, and *QPT*) and nicotine transport (i.e., *MATE1*) is reduced along with that of *NtERF189*. *NtERF189* belongs to subclade 2-1 of the Group IX AP2/ERF subfamily, which includes *NtORC1/ERF221*, and is highly homologous to *AtERF1* or *CrORCAs*. Interestingly, the *NIC2* locus contains seven clustered AP2/ERF genes, members of subclades 2-1 and 2-2 in the Group IX AP2/ERF subfamily.

Transgenic hairy roots with RNA interference (RNAi) of *NtERF189* using highly homologous regions in subclade 2 AP2/ERF genes showed a significant reduction in the transcript levels of nicotine biosynthetic enzyme genes (i.e., *PMT*, *N-methylputrescine oxidase (MPO)*, *AOX*, *QS*, *QPT*, and *A622*), and transporter genes (i.e., *MATE1/2*), whereas the transcript levels of *ODC*, *arginine decarboxylase (ADC)*, and *spermidine synthase (SPDS)* were not affected. Chimera repressor of *NtERF189* with an ERF-associated amphiphilic repression (EAR) motif at the C-termini showed similar results, except for the expression of *ODC*. Whereas the biosynthesis of nicotine and its biosynthetic enzyme genes in tobacco hairy roots were highly induced by MeJA, *NtERF189-RNAi* and *NtERF189-EAR* transformation suppressed MeJA-induced nicotine biosynthesis. Marked increases in the transcript levels of *PMT* and *QPT* and alkaloid production in hairy roots that overexpressed *NtERF189* without MeJA-treatment confirmed the transactivation activity of *NtERF189*. On the other hand, the overexpression of *NtERF179*, which belongs to subclade 2-2, marginally up-regulated the expression of *PMT* and *QPT* and increased the alkaloid content in both untreated and MeJA-treated conditions (Shoji et al., 2010), indicating that the ERFs of each subclade of the Group IX AP2/ERF subfamily have different functions, like ORCAs in MIA biosynthesis.

The direct binding of *NtERF189* to GCC-box in the *PMT* promoter and transactivation activity were also confirmed by an electrophoretic mobility shift assay (EMSA) and transient reporter assay with the *PMT* promoter. Whereas the *NIC2* locus ERF genes were substantially expressed in roots, some were expressed in other organs, particularly leaves. Importantly, they were not responsive to an ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC). In conclusion, AP2/ERF transcription factors at the *NIC2* locus are multiple positive regulators in nicotine biosynthesis which play a role in the JA-signaling cascade, although they may have different functions.



An AP2/ERF transcription factor, NbERF1, was also isolated in *N. benthamiana* (Todd et al., 2010). Whereas the amino acid similarity of NbERF1 to NtORC1/ERF221 suggests that the former may also play a role in nicotine biosynthesis, a detailed analysis has not yet been performed. An AP2/ERF family transcription factor has not yet been reported in BIA biosynthesis.

## 2.2 WRKY transcription factors

Whereas WRKY transcription factors are some of the major regulators in defense responses, the first WRKY was recently isolated in BIA biosynthesis from cultured *C. japonica* cells, which mainly produce berberine-type alkaloids (Kato et al., 2007) (see BIA biosynthetic pathway in Fig. 3). Kato et al. (2007) used expressed sequence tag (EST) information from high-berberine-producing cultured cells (156-1 cells) and an effective gene-silencing system with double-stranded (ds) RNA, i.e., transient RNAi in *C. japonica* protoplasts (Sato and Yamada, 1984, Dubouzet et al. 2005). The effects of silencing of candidate transcription factors in berberine biosynthesis in *C. japonica* protoplasts were monitored by measuring the transcript level of biosynthetic *norcoclaurine 6-O-methyltransferase (6OMT)* after the introduction of dsRNA. Transient RNAi of *CjWRKY1* clearly decreased the expression of berberine biosynthetic enzyme genes: *tyrosinedecarboxylase (TYDC)*, *norcoclaurine synthase (NCS)*, *6OMT*, *coclaurine-N-methyltransferase (CNMT)*, *(S)-N-Methylcoclaurine 3'-hydroxylase (CYP80B2)*, *3'-Hydroxy-N-methylcoclaurine-4'-O-methyltransferase (4'OMT)*, *berberine bridge enzyme (BBE)*, and *canadine synthase (CYP719A1)*. On the other hand, it did not affect the expression of genes that are not involved in berberine biosynthesis: *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, *3-deoxy-D-arabino heputulosonate 7-phosphate synthase (DAH7PS)*, *dehydroquinase shikimate dehydrogenase (DQSDH)*, and *chorismate mutase (CM)* in the biosynthesis of a berberine precursor, tyrosine. Overexpression of *CjWRKY1* enhanced the expression of transcript levels of the above-mentioned berberine biosynthetic enzyme genes, but did not affect those of primary metabolism genes. These results confirmed that *CjWRKY1* plays a role as a specific and general transcriptional activator in berberine biosynthesis in *C. japonica* cells (Kato et al., 2007). Direct binding to W-box of the promoter of biosynthetic enzyme genes and rapid MeJA-responsiveness were also observed for *CjWRKY1* (our unpublished data), which belongs to the Group IIc subfamily.

>> Figure 3

More recently, WRKY transcription factor has been reported in MIA biosynthesis (Fig. 1). Through the use of degenerate PCR primers that were designed based on conserved WRKY domains, several *WRKY* genes were isolated from MeJA-elicited *C. roseus* tissues by degenerate PCR and *CrWRKY1* that belonged to the Group III subfamily (Suttipanta et al., 2011). *CrWRKY1* was preferentially expressed in roots and showed responsiveness to MeJA, ethylene (ET), and gibberellic acid (GA). When the *CrWRKY1* gene was overexpressed in hairy roots, the expression of *TDC* was drastically increased, while the expression of *STR*, *G10H*, and *CPR* was not altered. Intriguingly, the overexpression of *CrWRKY1* resulted in the suppression of both the *ORCA2* and *ORCA3* genes. *CrWRKY1* could directly bind to W-box in the *TDC* promoter. These results suggest that *CrWRKY1* acts as an activator of the *TDC* gene and might activate another repressor that controls the expression of *ORCA* genes. Moreover, the accumulation of serpentine was significantly increased in hairy roots that overexpressed *CrWRKY1* compared to the control, whereas that of catharanthine was significantly decreased (Suttipanta et al., 2011). Based on these results, Suttipanta et al. (2011) speculated that *CrWRKY1* preferentially regulates the serpentine biosynthetic pathway in roots by activating *TDC* as well as repressing *ORCA* genes, while *ORCA3* preferentially controls the catharanthine biosynthetic pathway. Since serpentine biosynthetic enzyme genes have not yet been identified, it is unclear whether *CrWRKY1* controls the expression of these genes, but this hypothesis is consistent with the high expression level of *CrWRKY1* in roots.

### 2.3 Basic Helix-Loop-Helix transcription factors

*CrMYC1* was the first bHLH transcription factor to be reported in alkaloid biosynthesis. Chatel et al. (2003) found the JA- and Elicitor-Responsive Element (JERE) in the *STR* promoter containing G-box as well as GCC-box. They performed yeast one-hybrid screening to isolate *ORCA* genes and identified a typical bHLH transcription factor, *CrMYC1*, which belongs to Group B. Expression of the *CrMYC1* gene was induced by treatment with elicitor and MeJA, similar to that of the *STR* gene, but the up-regulation of *CrMYC1* was slower than that of *STR* (Chatel et al., 2003). These data suggest that *CrMYC1* may not control *STR* gene expression in response to elicitor or MeJA, although *CrMYC1* was shown to be able to bind to the G-box sequence in the *STR* promoter. Further experiments are necessary to confirm that *CrMYC1* plays a role in the regulation of MIA biosynthesis.

Recently, several groups have reported that bHLH, which is homologous to



*Arabidopsis* MYC2, is involved in controlling nicotine alkaloid biosynthesis (Todd et al., 2010; Zhang et al., 2011, 2012; Shoji and Hashimoto, 2011a)(Fig. 2). To isolate novel regulatory genes involved in nicotine alkaloid biosynthesis, Todd et al. (2010) performed two-step functional screening, similar to the procedure Kato et al. (2007) used in BIA biosynthesis; first, they created a cDNA library from MeJA-treated *N. benthamiana* roots and omitted cDNAs from MeJA-untreated leaves to isolate novel transcription factor genes. Next, they suppressed the expression of these genes by Virus-induced gene silencing (VIGS) while monitoring the nicotine content of leaves after treatment with MeJA. This screening revealed that the RNA-silencing of six genes affected nicotine levels in comparison to those in control plants. The suppression of two bHLH transcription factor genes, called *NbbHLH1* and *NbbHLH2* based on their similarity to *Arabidopsis* MYC2, significantly reduced the leaf nicotine content in MeJA-treated plants. RNA-silencing of *NbbHLH1* and *NbbHLH2* in transgenic plants significantly decreased the expression levels of *PMT*, *MPO*, *A622*, *QPT*, *AOX*, *QS* and *ODC*. In contrast, the overexpression of *NbbHLH1* and *NbbHLH2* slightly increased the nicotine content in transgenic plants. While the expression of *PMT* and *MPO* was slightly increased, the transcripts of other nicotine enzyme genes were not altered compared to those in control plants. The lower levels of nicotine and enzyme gene transcripts in plants that overexpressed *NbbHLHs* indicated that other regulatory components might be necessary for the regulation of MeJA-induced nicotine biosynthesis (Todd et al., 2010).

*NbbHLH1* could directly interact with G-box in the *PMT* promoter. The GCC-box, the target of NtORC1/ERF221, was proximal to this G-box. *NbbHLH1* and NtORC1/ERF221 cooperatively regulated the expression of *PMT* by interacting with G-box and GCC-box, respectively, as mentioned above. Furthermore, a yeast two-hybrid analysis showed that *NbbHLH1* could interact with NtJAZ1 and AtJAZ1, while NtORC1/ERF221 could not. AtMYC2 could also interact with NtJAZ1, suggesting that the JAZ-interacting domains of AtMYC2 homologues are highly conserved among plants, and the JA-mediated mechanism for the regulation of nicotine alkaloid biosynthesis by *NbbHLH1* might be similar to that of the AtMYC2-JAZ complex (De Boer et al., 2011).

*NtMYC2* genes (*NtMYC2a* and *NtMYC2b*), which are highly homologous to *NbbHLH1* and *NbbHLH2*, were also isolated from *Nicotiana tabacum*. RNA-silencing of *NtMYC2a* and *NtMYC2b* decreased the transcript levels of nicotine biosynthetic genes; *PMT*, *MPO*, *A622*, *QPT*, *AOX*, *QS*, *ODC*, berberine bridge enzyme-like (*BBL*), and *MATE1/2*, whereas the expression of *ADC* and *SPDS*, which is not

MeJA-responsive, was not altered (Shoji and Hashimoto, 2011a; Zhang et al., 2012). Furthermore, the suppression of *NtMYC2b* clearly decreased the expression levels of AP2/ERF genes at the *NIC*-locus (Shoji and Hashimoto, 2011a). These findings indicate that NtMYC2 directly induces the expression of nicotine biosynthetic genes, whereas it also affects them indirectly through regulation of the *NIC2*-locus AP2/ERF gene. Unlike the synergistic effects of NbbHLH1 and NtORC1/ERF221 on the expression of *PMT* and *QPT*, the co-expression of both *NtMYC2* and *NtERF189* resulted in only additive induction of the *PMT* and *QPT* genes. The interaction of NtMYC2 with NtJAZ1 was confirmed by a yeast two-hybrid analysis and a bimolecular fluorescence complementation (BiFC) analysis, which suggests that NtMYC2 forms a regulatory complex with NtJAZ1 and controls the expression of both nicotine biosynthetic genes and the other transcription factor genes in JA-signaling (Shoji and Hashimoto, 2011a; Zhang et al., 2012).

Recently, another bHLH transcription factor in MIA biosynthesis has been isolated from *C. roseus* (Zhang et al., 2011)(Fig. 1). This bHLH protein, distinct from CrMYC1, was homologous to NbbHLH1 and NtMYC2, and was designated CrMYC2. The *CrMYC2* gene was rapidly induced by treatment with MeJA. CrMYC2 could bind to the G-box-like element in the *ORCA3* promoter and activate the expression of the *ORCA3* promoter. RNA-silencing of *CrMYC2* significantly decreased both the transcript level of *ORCA3* in cultured *C. roseus* cells and MeJA-elicited MIA accumulation, while there was only a slight reduction in the transcript levels of *STR* and *TDC*. The overexpression of *CrMYC2* also induced the expression of both *ORCA2* and *ORCA3*. These results indicate that CrMYC2 is an activator of *ORCA3* gene expression mediated by MeJA. Interestingly, CrMYC2 did not up-regulate the expression of the *STR* promoter directly, whereas NtMYC2 did (Zhang et al., 2011).

In BIA biosynthesis, AtMYC2-type bHLH transcription factors have not yet been characterized. However, the non-AtMYC2-type bHLH transcription factor CjbHLH1 was isolated in berberine biosynthesis by transient RNAi screening from *C. japonica*, as described for *CjWRKY1* (Yamada et al., 2011a)(Fig. 3). Interestingly, a BLAST search of the EST database with the *CjbHLH1* sequence revealed that highly homologous genes were only found in the ESTs of BIA-producing plants (e.g., *Aquilegia formosa*) except for *Platanus occidentalis*. The phylogenetic tree based on the bHLH domain of several plants showed that CjbHLH1 and its homologues belonged to a distinct from AtMYC2-type transcription factors or bHLH proteins involved in phenylpropanoid biosynthesis (Yamada et al., 2011a). These findings suggest that CjbHLH1 might be specific to BIA biosynthesis.

The suppression of *CjbHLH1* expression by transient RNAi in 156-S protoplasts clearly decreased the expression of berberine biosynthetic enzyme genes (i.e., *TYDC*, *NCS*, *6OMT*, *CNMT*, *CYP80B2*, *4'OMT*, *BBE*, (*S*)-*Scoulerine-9-O-methyltransferase* (*SMT*), and *CYP719A1*), whereas the expression of primary metabolism genes (i.e., *GAPDH*, *DAH7PS*, *DQSDH*, and *CM*) or stress response genes (i.e., *pathogenesis-related protein 10* (*PR10*), *RAR1* (*required for Mla12 resistance*), *superoxide dismutase* (*SOD*), and *defensin*) was not affected. The overexpression of *CjbHLH1* modestly increased the expression of the above-mentioned biosynthetic enzyme genes, while the transcript levels of primary metabolism and stress response genes were not altered. These results indicate that *CjbHLH1* acts as a transcriptional activator of berberine biosynthetic enzyme genes and other transcription factors are required for the regulation of berberine biosynthesis by *CjbHLH1*, similar to *NbbHLHs* in nicotine biosynthesis (Yamada et al., 2011a).

## 2.4 Other transcription factors

The upstream region of the *STR* promoter contains another elicitor-responsive element (Menke et al., 1999a). With the use of this cis-element, the *CrBPF-1* gene was isolated by yeast one-hybrid screening (van der Fits et al., 2000). *CrBPF-1* is highly homologous to parsley box P-binding factor-1 (*PcBPF-1*) in the wound-induced defense response (da Costa e Silva et al., 1993), and contains a single MYB-like DNA-binding domain at the C-terminal end. The binding activity of *CrBPF-1* to the *STR* promoter was confirmed by EMSA and DNase I footprinting analyses. The *CrBPF-1* gene was significantly induced by elicitor, but not by MeJA. These findings indicate that *CrBPF-1* functions in JA-independent elicitor signaling. Furthermore, elicitor-induced *CrBPF-1* expression was significantly blocked by inhibitors of plasma membrane calcium channel (e.g., lanthanum chloride, gadolinium chloride, and nifedipine) and protein kinase inhibitors (e.g., K-252a and staurosporine), suggesting that *CrBPF-1* acts downstream of protein phosphorylation and calcium influx (van der Fits et al., 2000). The transcriptional activity of *CrBPF-1* was also confirmed by a transient reporter assay. *CrBPF-1* activated expression of the *STR* promoter, but its activity was weak compared to that of *ORCA2*. In addition, the combination of *CrBPF-1* and *CrMYC2* did not alter the expression level of the *STR* promoter, indicating that *CrBPF-1* does not interact with *CrMYC2* (Zhang et al., 2011).

As mentioned above, the G-box sequence in the *STR* promoter is a target of *CrMYC1*. On the other hand, EMSA analysis showed that tobacco G-box binding factor (*GBF*), *TAF-1*, could bind to this G-box (Pasquali et al., 1999). Through the use of this

TAF-1 sequence, two *CrGBF* genes (*CrGBF1* and *CrGBF2*) were isolated from a *C. roseus* cDNA library (Sibéril et al., 2001). Both *CrGBF1* and *CrGBF2* contain a basic-region leucine zipper (bZIP) motif at the C-terminus and a putative transactivation domain at the N-terminus. Similar to general bZIP transcription factors, both *CrGBFs* contain nuclear localization signals in the basic region. An EMSA analysis showed that both *CrGBFs* could bind not only to the G-box sequence in the *STR* promoter but also to the G-box-like sequence in the *TDC* promoter. A transient reporter assay showed that both *CrGBF1* and *CrGBF2* repressed the transcription of *STR* promoter through interaction with the G-box sequence (Sibéril et al., 2001).

The *TDC* promoter contains an elicitor-responsive region, with which ORCA transcription factors or CrBPF-1 could not interact (Ouwerkerk and Memelink, 1999). With this region of the *TDC* promoter, three TFIIIA-type zinc finger protein genes (named *ZCT1*, *ZCT2*, and *ZCT3*, for zinc finger Catharanthus transcription factor) were isolated by yeast one-hybrid screening (Pauw et al., 2004). These proteins contain a deduced repression domain found in most TFIIIA-type zinc fingers or several AP2/ERF transcription factors (Ohta et al., 2001). EMSA analysis demonstrated that these proteins could bind to the *TDC* and *STR* promoters. When the *TDC* and *STR* promoters were divided into several regions, *ZCT* proteins could interact with multiple regions that overlapped the target site of ORCA transcription factors. The overexpression of *ZCT* genes in cultured *C. roseus* cells clearly reduced *STR* and *TDC* promoter activity, suggesting that *ZCT* proteins act as transcriptional repressors of the *STR* and *TDC* promoters. Furthermore, the co-expression of *ZCT* with ORCA indicates that *ZCT* significantly suppressed the ORCA-induced expression of *STR* promoter through binding to *STR* promoter regions, which is distinct from a target site of ORCA proteins. Three *ZCT* genes were induced by MeJA and yeast extract as rapidly as *ORCA* genes were induced. The simultaneous induction of *ZCT* repressors and *ORCA* activators by MeJA- or elicitor-treatment suggests that the expression of MIA biosynthesis is fine-tuned spatio-temporally (Pauw et al., 2004).

A JA-responsive element (JRE) was found in the *ORCA3* promoter by an analysis of a series of *ORCA3* promoter-*GUS* fusion constructs. With the use of JRE, several protein genes that contained a DNA-binding AT-hook motif were isolated from *C. roseus* by yeast one-hybrid screening (Vom Endt et al., 2007). Interestingly, these AT-hook proteins could activate the expression of *ORCA3* promoter through specific binding to the JRE region, but the expression of *AT-hook* genes was not induced by MeJA-treatment (Vom Endt et al., 2007).

### 3. Upstream signals: Jasmonate and its signaling cascade

#### 3.1. Jasmonate cascade in a non-alkaloid-producing model plant, *Arabidopsis thaliana*

As mentioned above, jasmonates (JAs) are key signaling molecules in biotic and abiotic stress responses in plants. The JA signaling pathway has been intensively studied in the model plant *Arabidopsis thaliana*. The JA-insensitive mutant *coi1*, which is resistant to coronatine, a bacterial compound that is structurally similar to JAs (Feys et al., 1994; Xie et al., 1998), is used to isolate the *COI1* gene. *COI1* encodes an F-box protein of Skp/Cullin/F-box (SCF<sup>COI1</sup>)-type E3 ubiquitin ligase in protein degradation by 26S proteasome. A deficiency of other components of SCF complexes, such as ASK1/2, AXR1, SGT1b, and COP9 signalosome, is also known to impair JA responses (Browse, 2009). Mutation in the *MYC2* gene, which encodes a bHLH transcription factor, is found in JA-insensitive *jail/jin1* mutants, and AtMYC2 has been reported to play an essential role in the JA signal pathway in the responses to wounding and insect attack (Lorenzo et al., 2004; Dombrecht et al., 2007) (Fig. 4A).

Further investigation identified the jasmonate ZIM domain (JAZ) in repressor proteins in JA signaling. JAZ interacts with the SCF<sup>COI1</sup> complex and is degraded by 26S proteasome in response to JA (Chini et al., 2007; Thines et al., 2007; Staswick, 2008). The ZIM domain of JAZ proteins interacts with different JAZ proteins in a homo- and heterodimeric manner (Chini et al., 2009) and recruits general transcriptional co-repressors TOPLESS (TPL) and TPL-related proteins through interaction with an adaptor protein, Novel Interactor of JAZ (NINJA) (Pauwels et al., 2010). JAZ proteins also directly interact with AtMYC2 and block its activity in the absence of JA. On the other hand, the presence of JAs, especially bioactive JA-Ile, induces the formation of a complex of JAZ proteins and SCF<sup>COI1</sup>, degradation by 26S proteasome, and the release of AtMYC2 for the expression of JA-responsive genes (Chico et al., 2008) (Fig. 4A).

>>Figure 4

Ethylene (ET) is another key mediator in the pathogenesis response and several AP2/ERF-domain transcription factor proteins have been identified in ethylene signaling (Memelink, 2009). It is now evident that JA and ET signaling pathways interact with each other and AP2/ERF-domain proteins play a role in this interaction (Fig. 4A). For example, the gene expression of an AP2/ERF-domain protein, ERF1 (At3g23240), in *A. thaliana* was rapidly induced by JA and ET in wild type (WT) plants, but the *coi-1* mutation abolished the induction by JA (Lorenzo et al., 2003). On the other hand, the overexpression of *ERF1* in the *coi-1* mutant induced

defense-response genes such as *Plant Defensin 1.2* (*PDF1.2*) and *basic Chitinase* (*b-CHI*) as in JA-treated plants. *Octadecanoid-Responsive Arabidopsis AP2/ERF 59* (*ORA59*) induced by MeJA and ET also acts as an activator of the expression of defense-related genes, such as *PDF1.2*, downstream of COI1 (Pré et al., 2008). ERF1 and ORA59 would function redundantly. AtERF1 (At4g17500, which is a different ERF than ERF1) and AtERF2 have been reported to play a role in the accumulation of the *PDF1.2* transcript in constitutively overexpressing plants (McGrath et al., 2005; Pré et al., 2008), whereas AtERF1 and AtERF2 did not activate the *PDF1.2* promoter in a transient activation assay (Pré et al., 2008; Zarei et al., 2011). It has been speculated that AtERF1 and AtERF2 may affect the expression of *PDF1.2* indirectly. AP2/ERF domain proteins consist of both activators and repressors. Whereas AtERF5 and AtERF6 function redundantly as positive regulators in JA-mediated plant defense (Moffat et al., 2012), AtERF4 with an EAR repressor motif suppressed the expression of *PDF1.2* under treatment with MeJA but enhanced this expression in its knockout mutants (McGrath et al., 2005).

Mitogen-activated protein kinase 6 (MPK6) and MAPK kinase 3 (MKK3) are negative regulators of the *AtMYC2* gene (Takahashi et al., 2007)(Fig. 4A). The MKK3-MPK6 cascade negatively regulates the expression of the *AtMYC2* gene. JA activates MPK6 through MKK3, which represses the expression of *AtMYC2*, but also directly activates the expression of *AtMYC2*. This negative and positive regulation by JA may be used to fine-tune JA signaling, while the repression of *AtMYC2* up- and down-regulates the expression of *PDF1.2* and *Vegetative Storage Protein* (*VSP*), respectively.

Two NAC (NAM/ATAF1,2/CUC2) transcription factors, ANAC019 and ANAC055, in *A. thaliana* are positive regulators of the *VSP* gene (Bu et al., 2008). MeJA-treatment induced the rapid induction of *VSP* in WT plants, whereas this was abolished by the *coi1* and *myc2* mutations. MeJA-induced *VSP* gene expression is dependent on ANAC019/ANAC055, since the *anac019 anac055* double mutation significantly decreased *VSP* expression, while the overexpression of ANAC019 or ANAC055 increased its expression under MeJA-treatment (Fig. 4A). The rescued induction of *VSP* expression by the overexpression of ANAC019 in *myc2* mutants under MeJA-treatment indicates that ANAC019 and ANAC055 act downstream of AtMYC2 in the JA signaling cascade (Bu et al., 2008).

Recently, another type of transcription factor, DELLA RGA-LIKE3 (RGL3), has also been reported to be involved in the regulation of defense-response genes in the JA signaling cascade (Wild et al., 2012)(Fig. 4A). DELLAs are transcription factors



that contribute to growth regulation in a gibberellic acid-mediated signaling pathway. MeJA transiently induced the expression of *RGL3*, and this induction was strongly reduced in the *coil* mutant and the triple *myc2 myc3 myc4* mutant. AtMYC2 controls *RGL3* expression through binding to *RGL3* promoter. Furthermore, fluorescence lifetime analyses and co-immuno-precipitation experiments directly indicate that RGL3 protein interacts with both JAZ1 and JAZ8 proteins. On the other hand, *rgl3* mutation attenuated the expression of JA-induced defense-response genes and made plants more susceptible to pathogens. These results indicate that the JA-mediated expression of *RGL3* plays an important role in the expression of JA-responsive genes and the defense response (Wild et al., 2012). It has been speculated that the release of AtMYC2 through the JA-induced degradation of JAZ proteins activates not only the expression of *JAZ* genes for positive feedback regulation, but also that of *RGL3* to enhance the competitive interaction of RGL3 with JAZ proteins. RGL3 might play an important role in modulating JA-mediated action by facilitating the responsiveness of plants to JA. Under favorable growth conditions, when GA biosynthesis is enhanced, the SCF<sup>SLY1</sup> complex degrades RGL3 by 26S proteasome, and the JA signaling response is compromised due to the accumulation of free JAZ proteins, which can associate with AtMYC2.

### 3.2. Signal cascade in nicotine biosynthesis (Fig. 4B)

*NaCOI1*, a homologue of *AtCOI1*, was isolated from *Nicotiana attenuate*, and stable transformants that showed a reduced expression of *NaCOI1* (*ir-coil*) were produced with RNAi-vector (Paschold et al., 2007). These *ir-coil* plants not only showed an insensitivity of root growth to MeJA-treatment and male sterility due to impaired anther dehiscence, but also lowered nicotine levels, trypsin proteinase inhibitor (TPI) activity, and caffeoylputrescine accumulation. Additional experiments showed that NaCOI1 plays an important role in the resistance to herbivores (Paschold et al., 2007).

A further RNA-silencing experiment with *NtCOI1* isolated from *N. tabacum* (Shoji et al., 2008) showed that the MeJA- and wound-induced expression of genes involved in nicotine biosynthesis (*PMT* and *QPT* genes) was suppressed, and confirmed that COI1 plays an important role in nicotine biosynthesis. The further characterization of three tobacco *JAZ* cDNAs (designated *NtJAZ1*, *NtJAZ2* and *NtJAZ3*), which contain highly conserved ZIM and Jas motifs, MeJA-induced gene expression, and their rapid degradation through proteasome after MeJA treatment, showed that JAZ was involved in nicotine biosynthesis. The inhibition of *PMT* and *QPT* gene expression by a

proteasome inhibitor, MG132, under JA-treatment, and the decrease in nicotine accumulation due to the expression of non-degradable C-terminal Jas-motif truncated proteins clearly indicate that the JA-induced degradation of JAZ repressors by the COI1-dependent 26S proteasome system is a key regulatory mechanism in nicotine biosynthesis (Shoji et al., 2008).

Further studies have shown that a mechanism similar to that in the JA signaling pathway in *Arabidopsis* participated in nicotine biosynthesis (Fig. 4B). Thus, AtMYC2 homologues (i.e., NbbHLH1, NbbHLH2, NtMYC2a and NtMYC2b) could interact with NtJAZ proteins, whereas NtERF189 could not interact with NtJAZs, suggesting that COI1-JAZs-MYC2 is involved in the regulation of nicotine biosynthesis in the response to JA (Shoji and Hashimoto, 2011a; Zhang et al., 2012). The degradation of JAZ proteins allows the binding of active MYC2 to a G-box sequence of the promoter of genes of biosynthetic enzymes, transporter and *NIC2*-locus AP2/ERF transcription factors, which additively or synergistically regulate the expression of biosynthetic genes (Fig. 4B). However, the ectopic expression of *NtMYC2a* and *NtMYC2b* in tobacco BY-2 cells had little effect on the expression of genes involved in nicotine biosynthesis (Zhang et al., 2012). Since NtMYC2 activates *JAZ* gene expression, the ectopic expression of *NtMYC2* might lead to the feedback repression of its own activity.

Protein phosphorylation is another mechanism for the regulation of JA signaling. A transient expression assay under co-treatment with MeJA and MAP kinase inhibitor indicated that protein phosphorylation alters NtORC1/ERF221 (*NIC2*-locus ERF)-mediated transcriptional activation of nicotine biosynthetic enzyme genes. The induction of *JA-factor stimulating MAPKK1 (JAM1)* by MeJA coincided with the expression of *NtORC1* and other biosynthetic enzyme genes in tobacco BY-2 cells (Goossens et al., 2003; De Boer et al., 2011). Whereas the overexpression of *JAM1* alone did not activate the expression of the *PMT* and *QPT* promoters, its co-overexpression with *NtORC1* dramatically increased the expression of the *PMT* and *QPT* promoters under MeJA treatment. This synergistic effect of *JAM1* expression was also found with *NbbHLH1*. These results clearly indicate that a MAP kinase phosphorylation cascade is involved in the regulation of nicotine biosynthesis through NbbHLH1 and NtORC1/ERF221. Whereas nicotine biosynthesis involves a regulatory mechanism similar to that seen in the JA-signaling pathway in *Arabidopsis*, it is possible that other protein kinases, which may affect the bHLHs, AP2/ERFs, WRKYs, NbARF1 and NbHB1, which are also induced by MeJA, might be involved in the regulation of nicotine alkaloid biosynthesis (Goossens et al., 2003; Todd et al., 2010).



### 3.3. Signal cascade in monoterpene-indole alkaloid (MIA) biosynthesis (Fig. 4C)

The MYC2-type transcription factor CrMYC2 modulates *ORCA* gene expression in MIA biosynthesis in *C. roseus*, as mentioned above. Whereas no JAZ or COI1 homologues have been reported to be involved in the regulation of MIA biosynthesis, several lines of evidence suggest that CrMYC2 may function as both JAZ and COI1 proteins in MIA biosynthesis. First, CrMYC2 is highly homologous to AtMYC2 and tobacco MYC2s, and contains a JAZ-interaction domain (JID) (Fernández-Calvo et al., 2011). Second, the overexpression of *CrMYC2* in cultured *C. roseus* cells was associated with only a slight increase in the gene expression of *ORCA3* and biosynthetic enzymes, similar to the overexpression of *NtMYC2a* and *NtMYC2b* in tobacco BY-2 cells (Zhang et al., 2011), which suggests the post-translational regulation of CrMYC2, probably through JAZ repressors, as seen with NtMYC2 (Fig. 4C). The isolation and characterization of COI1 and JAZ proteins from *C. roseus* should provide more information about the mechanism by which the CrMYC2-JAZ-COI1 complex regulates MIA biosynthesis. One difference from nicotine biosynthesis is that CrMYC2 cannot directly regulate the expression of MIA biosynthetic enzyme genes, but rather activates the expression of *ORCA* genes, and thereby the expression of several MIA biosynthetic enzyme genes. Whereas JA can control *ORCA* expression through the CrMYC2-JAZ-COI1 core complex, and thereby MIA biosynthesis, other transcription factors may also play a role in MIA biosynthesis, since *ORCA* alone is not sufficient to regulate some enzyme genes in the pathway, such as *G10H* and *DAT*.

In addition to *ORCA*s and *MYC2*, *ZCT*s (Zinc-finger *C. roseus* transcription factors) and *WRKY1* have been shown to exhibit JA-responsiveness in MIA biosynthesis in *C. roseus* (De Geyter et al., 2012). *ZCT* proteins are transcriptional repressors in MIA biosynthesis, and, like *ORCA* genes, are rapidly induced by MeJA. Although the functional relationship between *ZCT* and CrMYC2/*ORCA*s has not yet been characterized, *ZCT* proteins might generate negative feedback loops without JAZ repressors to balance the expression of biosynthetic genes in the JA-signaling pathway. *CrBPF-1* and *CrGBF1/2*, which are not induced by MeJA, have also been reported (van der Fits et al., 2000; Sib  ril et al., 2001).

CrWRKY1 acts as a transcriptional activator in *C. roseus*, since the overexpression of *CrWRKY1* in hairy roots markedly increased the expression of *TDC*. On the other hand, the overexpression of *CrWRKY1* also increased the transcript levels of *ZCT1*, *ZCT2* and *ZCT3*, which act as repressors in MIA biosynthesis, and decreased the transcript levels of *ORCA2*, *ORCA3* and *CrMYC2* (Suttipanta et al., 2011). These positive and negative effects of CrWRKY1 on MIA biosynthesis reflect the complexity

of MIA biosynthesis, which is regulated spatially and temporally, and both positive and negative transcription factor genes are induced by treatment with JA.

Protein phosphorylation has also been suggested to be involved in the regulation of MIA biosynthetic genes, since treatment with K-252a and staurosporine significantly decreased the elicitor- and MeJA-induced expression of the *STR* and *TDC* genes (Menke et al., 1999b). More recently, *C. roseus* protein kinase 3 (CrMPK3) was found to regulate MIA biosynthesis (Raina et al., 2012). CrMPK3 is highly homologous to AtMPK3, which is involved in the response to biotic and abiotic stresses as well as developmental processes. MeJA rapidly induced the expression of *CrMPK3* and promoted its own phosphorylation in *C. roseus* plants. Whereas the CrMPK3-GFP fusion protein was localized in the cytoplasm under normal conditions, treatment with MeJA induced translocation to nucleus. Transient overexpression of *CrMPK3* in leaves activated several MIA biosynthetic enzyme genes and the *ORCA3* activator and moderately suppressed *ZCT* repressors. The overexpression of *CrMPK3* in leaves induced the accumulation of several MIAs, such as serpentine, vindoline and vincristine (Raina et al., 2012). These results indicate that the MAPK signal cascade including CrMPK3 also plays a role in MIA biosynthesis in the JA signal pathway. Whereas the targets of CrMPK3 have not yet been identified, the recent finding that AtMPK3 phosphorylates AtWRKY33 in camalexin biosynthesis (Ren et al., 2008; Mao et al., 2011) might be informative.

### 3.4. Signal cascade in benzyloquinoline alkaloid (BIA) biosynthesis (Fig. 4D)

While the role of an MYC2-type bHLH transcription factor has not yet been determined, non-MYC2 type CjbHLH1, a transcriptional activator in berberine biosynthesis, has been isolated from *C. japonica*. However, overexpression of the *CjbHLH1* gene was rapidly induced by MeJA, and it has been suggested that CjbHLH1 is involved in the JA signaling cascade (Yamada et al., 2011b). Interestingly, while MYC2-type bHLHs contain a bHLH domain at the C-terminal end, this domain is located at the N-terminal end in CjbHLH1. Additionally, CjbHLH1 lacks a JID, which is found in the N-terminal half of MYC2 which suggests that CjbHLH1 cannot interact with JAZ proteins and function without the formation of a complex between JAZ and COI1 in the JA signaling pathway. However, the recent isolation of full cDNA of *C. japonica* MYC2 and three JAZ genes (our unpublished data), suggests that a MYC2-COI1-JAZ core complex may participate in the JA signaling cascade even in *C. japonica*, while COI1 has not yet been isolated. It would be interesting to determine how the CjMYC2-COI1-JAZ complex contributes to the regulation of berberine

biosynthesis, and how CjbHLH1 is involved in the JA signaling cascade with or without this complex.

Expression of the *CjWRKY1* gene was rapidly induced by MeJA in BIA biosynthesis in cultured *C. japonica* cells, similar to that of *CjbHLH1* (our unpublished data), which suggests that CjWRKY1 plays a role in the JA signaling pathway. Transient RNA-silencing of *CjbHLH1* did not affect the expression of *CjWRKY1*, and vice versa (our unpublished data). CjbHLH1 and CjWRKY1 may act independently in the BIA biosynthetic pathway in *C. japonica*. While the mechanism that regulates JA-induced expression of the *CjbHLH1* and *CjWRKY1* genes is still unknown, the involvement of the MYC2-COI1-JAZ core complex as well as phosphorylation should be examined in the near future (Fig. 4D).

#### 4. Evolution of regulatory mechanisms in secondary metabolism

As mentioned in the Introduction, plants produce a wide array of low-molecular-weight metabolites, i.e., secondary metabolites including alkaloids, in response to abiotic and biotic stress. Here, we compare the regulatory networks for the biosynthesis of different types of metabolites, i.e., phenylpropanoids, terpenoids, and alkaloids. Information regarding alkaloid biosynthesis is considerably limited in comparison with the other pathways, especially that of phenylpropanoids.

##### 4.1. Regulatory mechanism in anthocyanin biosynthesis

Phenylpropanoids are most commonly found in higher plants and their biosynthetic pathways and transcriptional regulation by transcription factors have been intensively investigated. Phenylpropanoids include anthocyanins, which are major flower pigments that are widely distributed in plant species, flavonoids, stilbenes, lignans/lignins and so on, and have important physiological roles in attracting insects to pollinate flowers and disperse seeds, protecting plants from UV irradiation, and acting as antimicrobial agents against insect attack and pathogen infection (Grotewold, 2006).

Flavonoid biosynthesis, like that of anthocyanins, starts with chalcone synthase (CHS), which catalyzes the condensation of malonyl-CoA with *p*-coumaroyl-CoA to produce naringenin chalcone. Chalcone isomerase (CHI) converts naringenin chalcone into naringenin. Flavanone 3-hydroxylase (F3H) and flavonoid 3'-hydroxylase (F3'H) produce flavanol by the hydroxylation of naringenin, from which dihydroflavonol reductase (DFR) produces leucoanthocyanidins. Leucoanthocyanidin dioxygenase (LDOX) converts leucoanthocyanidins to anthocyanins. UDP-3-*O*-glucosyltransferase (UGT) is involved in the glycosylation of anthocyanins (Grotewold, 2006).

In anthocyanin biosynthesis, the WD40-MYB-bHLH complex acts as an important regulatory module (Koes et al., 2005) (Fig. 5). The WD40-repeat protein, Transparent Testa Glabra 1 (TTG1), recruits bHLH transcription factors, such as Glabra 3 (GL3), Enhancer of Glabra 3 (EGL3) and Transparent Testa 8 (TT8), as well as MYB transcription factors, such as Production of Anthocyanin Pigment 1 (PAP1)/MYB75, PAP2/MYB90, MYB113 and MYB114, to form a regulatory complex, and modulates the expression of the 'late' anthocyanin biosynthetic enzyme genes including *DFR*, *LODX* and *UGT* in *A. thaliana*. This regulatory machinery of the WD40-MYB-bHLH complex is widely distributed in phenylpropanoid biosynthesis (Grotewold, 2006).

Developmental signals, sugar, and environmental stresses, such as UV light, temperature, drought, wounding, and pathogen infection, are known to control

anthocyanin biosynthesis. Plant hormones also regulate anthocyanin biosynthesis. For instance, benzyladenine (BA), a synthetic cytokinin, induces the accumulation of anthocyanin by modulating the expression of *CHS* and *DFR* in Arabidopsis plants (Deikman and Hammer, 1995), whereas GA represses the sucrose-induced expression of anthocyanin biosynthesis genes and decreases anthocyanin content (Loreti et al., 2008).

MeJA also enhances pigmentation in plants (Tamari et al., 1995), and the presence of sucrose under treatment with JA significantly up-regulates the expression of anthocyanin biosynthetic enzyme genes such as *DFR*, *LODX* and *UF3GT*, and leads to an increased accumulation of anthocyanin (Loreti et al., 2008; Shan et al., 2009). The suppression of the expression of biosynthetic enzyme genes and the accumulation of anthocyanins in *coil* mutants suggests that COI1 plays a role in JA-mediated anthocyanin biosynthesis. Note that the expression of transcription factor genes including *PAP1*, *PAP2* and *GL3* was also increased in JA-stimulated plants. Although the detailed mechanism of the interaction of sucrose signaling and hormone signaling pathways remains unknown, JA-regulated transcription factors may modulate the expression of several biosynthetic enzyme genes and the accumulation of anthocyanins (Shan et al., 2009).

It has recently been shown that JAZ repressor proteins participate in JA-mediated anthocyanin biosynthesis (Fig. 5). JAZ proteins interact with bHLH transcription factors, such as *GL3*, *EGL3* and *TT8*, and *R2R3-MYB* transcription factors, *PAP1/MYB75* and *Glabra 1 (GL1)* (Qi et al., 2011). They interact with each other through their C-terminal domain and Jas motif, which is involved in the dimerization of JAZ proteins. JAZ proteins also affect the interaction of bHLHs with MYBs and the initiation of trichomes. *coil* mutants that had been treated with JA showed no accumulation of anthocyanin and a decrease in trichomes, whereas the overexpression of *PAP1* rescued this defect in anthocyanin biosynthesis. When *GL3* or *EGL3* was overexpressed in *coil* mutants, trichome formation was dramatically increased, but anthocyanin accumulation was modest, suggesting that *GL3* or *EGL3* mediates JA-induced anthocyanin biosynthesis and trichome formation via different downstream signaling cascades (Qi et al., 2011). These results suggest that JAZ repressors regulate the activity of the WD40-MYB-bHLH complex in the JA signaling cascade and the JA-induced degradation of JAZ proteins by the SCF<sup>COI1</sup> complex releases WD40-MYB-bHLH regulatory complexes to modulate the expression of their respective downstream genes.

This mechanism for the regulation of COI1-JAZ-WD40-MYB-bHLH in anthocyanin biosynthesis would be similar to that of the COI1-JAZ-MYC2 core

complex involved in the defense response, and nicotine or MIA biosynthesis (Fig. 5). A central player, in the JA-mediated signaling pathway, the COI1-JAZ receptor complex, may be conserved in the defense responses in many plant species and used in the production of various secondary metabolites. The difference in the partners that interact with the COI1-JAZ complex would determine the sets of genes for transcription factors and biosynthetic enzymes that are involved in different secondary metabolite pathways.

>>Figure 5

#### 4.2 Regulatory mechanism in sesquiterpenoid biosynthesis

Terpenoids are another large class of secondary metabolites that play a diverse role in plant-microbe, plant-insect, plant-plant and plant-environment interactions (Pichersky and Gershenzon, 2002). All terpenoids are synthesized from isoprene (C<sub>5</sub>) units. Sesquiterpenes (C<sub>15</sub>), which consist of three isoprene units, are defense-response chemicals, so-called ‘phytoalexins’. Several transcription factors involved in sesquiterpene biosynthesis have been characterized.

Whereas *A. thaliana* does not produce alkaloids, its flowers emit some sesquiterpenes, such as (*E*)-caryophyllene, (+)-thujopsene, α-humulene, (*E*)-β-farnesene, (+)-β-chamigrene and (-)-cuparene (Chen et al., 2003). Recently, the positive regulation of the biosynthesis of these sesquiterpenes by AtMYC2 was characterized (Hong et al., 2012). The *myc2* mutation significantly reduced the emission of sesquiterpenes and the expression of *terpene synthase* (*TPS*) genes, i.e., *TPS11* and *TPS21*, in comparison with WT. On the other hand, plants that overexpressed AtMYC2 emitted more sesquiterpenes and showed a higher expression of *TPS11* and *TPS21* than WT plants. Direct binding of AtMYC2 to the E-box element in the *TPS11* and *TPS21* promoters has also been demonstrated. Interestingly, MeJA and GA significantly increased the expression of *TPS11* and *TPS21* in WT inflorescences and the *myc2* mutation abolished the effect of phytohormones. Overexpression of a DELLA protein (i.e., RGA), a negative regulator of GA signaling, also markedly reduced the expression of *TPS11* and *TPS21*, whereas the penta *della* mutant was more sensitive to MeJA. The direct interaction of RGA with AtMYC2 has also been reported to competitively affect the interaction between AtMYC2 and JAZs (Hong et al., 2012). These results suggest that AtMYC2 controls sesquiterpene biosynthesis in a JA-responsive manner through its interaction with GA-signaling, especially through the competitive interaction of JAZ and DELLA proteins in the JA and GA signaling cascades (Fig. 5). As



mentioned above with regard to phenylpropanoid biosynthesis, MYC2 may be a key regulator in plant defense responses. The importance of MYC2 in the evolution of secondary metabolism will be discussed below.

In addition to MYC2, a WRKY transcription factor, GaWRKY1, was identified in the biosynthesis of gossypol and other sesquiterpenes of *Gossypium arboreum* (Xu et al., 2004). GaWRKY1 belongs to the Group IIa subfamily and MeJA rapidly induces the gene expression of *GaWRKY1* and regulates the transcription of (+)- $\delta$ -*cadinene synthase 1* (*CAD1*), a sesquiterpene cyclase that is involved in gossypol biosynthesis.

Another WRKY transcription factor, AaWRKY1, was isolated in the biosynthesis of an anti-malarial sesquiterpene lactone endoperoxide, artemisinin, from *Artemisia annua* (Ma et al., 2009). Artemisinin has also recently been reported to have antiviral and anticancer activities. AaWRKY1 is a member of the Group III WRKY subfamily and treatment with MeJA and chitosan rapidly increases the gene expression of *AaWRKY1*. AaWRKY1 directly binds to the W-box sequence of *amorpho-4*, an *11-diene synthase* (*ADS*) gene promoter, and activates its expression, suggesting that AaWRKY1 acts as an activator in artemisinin biosynthesis. Recently, novel AP2/ERF transcription factors, AaERF1 and AaERF2, which belong to the Group IX subfamily, were also isolated in artemisinin biosynthesis (Yu et al., 2012). Transient induction of the expression of *AaERF1* and *AaERF2* by MeJA, the direct binding of AaERF1/2 to the *ADS* and *CYP71AV1* promoters and their activation, and the enhanced accumulation of artemisinin in transgenic *A. annua* leaves that overexpress either *AaERF1* or *AaERF2* indicate that both AaERF1 and AaERF2 function as positive regulators in artemisinin biosynthesis.

These WRKY proteins in sesquiterpene biosynthesis showed JA-responsiveness, similar to WRKY proteins identified in the biosynthesis of BIA (i.e., CjWRKY1) and MIA (i.e., CrWRKY1), suggesting that WRKY proteins might be common mediators in defense responses in the JA signaling cascade, whereas WRKY proteins in phenylpropanoid biosynthesis have been less characterized. Interestingly, the four WRKY proteins apparently have different structures. Whereas both CrWRKY1 and AaWRKY1 belong to the Group III subfamily, GaWRKY1 and CjWRKY1 are classified into the Group IIa and IIc subfamilies, respectively. These differences might be due to distinct post-translational regulation, including protein-protein interaction and modification. Isolation of the WRKY proteins involved in nicotine biosynthesis would be needed.

On the other hand, the AP2/ERF transcription factors that have been identified in the regulation of sesquiterpene and alkaloid biosynthesis are all JA-responsive and

classified into the Group IX subfamily, indicating that the transcription of this Group IX AP2/ERF might have acquired a specific function in the regulation of sesquiterpene and alkaloid biosynthesis during evolution. It would be interesting to know whether AP2/ERF transcription factors function in the biosynthesis of BIA or other secondary metabolites via a JA signaling pathway. The interaction of AP2/ERF transcription factors and other transcription factors (bHLHs) might be different in the biosynthesis of different types of alkaloids, as has been observed for NtMYC2 and ERF189 in nicotine biosynthesis and CrMYC2 and ORCA2/3 in MIA biosynthesis.

#### 4.3. Evolution of regulatory mechanism in alkaloid biosynthesis and gene clustering

It is generally believed that secondary metabolism is derived from primary metabolism by gene duplication and possibly by allelic divergence (e.g., Shoji and Hashimoto, 2011b). Furthermore, mutations in biosynthetic enzyme genes and the consequent deletion or gain of a novel metabolic pathway, transporter genes necessary for the compartmentalization of cytotoxic metabolites, and modified gene expression profiles of transcriptional regulators contribute to the diversification of secondary metabolism (Liscombe et al., 2005, Takemura et al., 2010). On the other hand, secondary metabolism is part of the environmental response, especially in plant defenses. Environmental stresses as well as plant defense mechanisms are diverse. Thus, the regulation of secondary metabolism may include both a common and specific regulation in each pathway.

JA signaling and plant MYC2-type transcription factors are key players in the responses to various external stimuli and universally function in the biosynthesis of many secondary metabolites, as discussed above. JAZ and DELLA proteins are also highly conserved in plants and are involved in the biosynthesis of many metabolites, including alkaloids (Fig. 5). The interaction of tobacco MYC2 in nicotine biosynthesis with non-alkaloid-producing *A. thaliana* JAZ proteins and the direct binding of AtMYC2 to the *ORCA3* promoter in MIA biosynthesis may reflect a conserved regulatory mechanism in secondary metabolism in plant defense (De Boer et al., 2011; Montiel et al., 2011). In this sense, it would be interesting to isolate MYC2-type transcription factors in BIA biosynthesis to understand the conservation and evolution of regulatory mechanisms in alkaloid biosynthesis.

On the other hand, it is possible that specific alkaloid-producing plants may have unique biosynthetic enzymes and transcription factors. BIA-specific cytochrome P450 enzymes, i.e., proteins in the CYP719 family, are known to play an important role in BIA biosynthesis (Fig. 3). CYP719A1, which converts (*S*)-tetrahydrocolumbamine



into (*S*)-canadine in *C. japonica*, CYP719A2/3/5, which converts (*S*)-scoulerine into (*S*)-stylopine via (*S*)-cheilanthifoline in *E. californica*, CYP719B1, which converts (*R*)-reticuline into salutaridine in *P. somniferum*, and other enzymes in the CYP719 family have been shown to participate in the biosynthesis of divergent BIAs (Ikezawa et al., 2003, 2007, 2009; Gesell et al., 2009). Similar to enzymes in the CYP719 family, a bHLH-type transcription factor, i.e., CjbHLH1, which is involved in BIA biosynthesis in *C. japonica* and its homologues, should also be specific for BIA-producing plant species. CjbHLH1 is distinct from MYC2-type bHLH transcription factors, which are key regulators in defense responses, including the production of many secondary metabolites. It would be interesting to study the unique distribution of CjbHLH1 and its homologues and enzymes in the CYP719 family in BIA-producing plant species to better understand the evolution of the biosynthetic pathways of structurally divergent BIAs.

Another interesting question in the evolution of secondary metabolism is the organization of the genes involved in biosynthesis. Whereas genes in phenylpropanoid biosynthesis are known to not form a gene cluster, a recent investigation revealed that some metabolites, such as thalianols in *A. thaliana*, benzoxazinoids in maize, avenacins in oat and momilactones in rice, formed clusters of genes for biosynthetic enzymes (Frey et al., 1997; Qi et al., 2004; Shimura et al., 2007; Field and Osbourn, 2008). Sequential gene duplications, genome rearrangements and gene loss presumably cause the formation of these gene clusters. The selective advantages of secondary metabolites, which confer resistance to pests and pathogens on plants, would also contribute to the formation of gene clusters.

For example, the production of a variety of diterpenoid phytoalexins, momilactones and phytocassanes in rice, is rapidly induced by pathogen attack. Momilactone biosynthetic enzyme genes are clustered on rice chromosome 4 and the expression of these genes is coordinately regulated by treatment with a fungal elicitor (Shimura et al., 2007). Okada et al. (2009) identified an elicitor-inducible basic leucine zipper (bZIP) transcription factor, OsTGAP1, by a comparative microarray analysis with elicited or non-elicited rice plants. *OsTGAP1* knockout mutants showed a low accumulation of momilactones under elicited conditions and a defect in the induction of biosynthetic gene transcripts. In contrast, transgenic plants that overexpressed *OsTGAP1* showed a higher accumulation of momilactones as well as phytocassanes and transcripts of biosynthetic genes than WT plants, even when non-elicited. Although it is not clear whether OsTGAP1 regulates the expression of clustered genes for momilactone biosynthesis through chromatin modifications or some as-yet-undefined

mechanisms, OsTGAP1 could be the master regulator of phytoalexin biosynthesis in rice via the coordinated regulation of clustered biosynthetic enzyme genes (Okada et al., 2009).

More recently, a gene cluster that consists of 10 biosynthetic enzyme genes within a 221 kb region has been reported in noscapine alkaloid biosynthesis in opium poppy (Winzer et al., 2012). A VIGS analysis showed that 6 of the 10 genes are involved in a noscapine biosynthetic pathway. The identification of gene clusters in alkaloid biosynthesis, as in other secondary metabolite pathways, indicates that gene clustering might not be rare. While genes in a cluster would be regulated in a coordinated manner by transcriptional regulators at the chromatin level, W-box elements, targets of WRKY transcription factors, were found within 1 kb deduced promoter regions upstream of the open reading frames of the 10 genes (Winzer et al., 2012), which suggests that WRKY transcription factor plays an important role in noscapine biosynthesis.

On the other hand, it would be interesting to study why the biosynthetic pathways of only a limited number of secondary metabolites form gene clusters, while the tight regulation of biosynthesis by the spatial and temporal expression of individual biosynthetic enzymes and of compartmentalization by the coordinated expression of specific transporters are indispensable. Further studies on the coordinated expression of biosynthetic genes, especially at the chromatin level, are needed.

## 5. Application of transcription factors to natural product engineering

Elucidation of the biosynthetic pathways of secondary metabolites enables us to improve the production yield and quality (Sato et al. 2007). Whereas the metabolite quality can be controlled by modifying metabolite profiles by introducing or suppressing a certain pathway, the production yield of metabolites can be improved by overexpression of genes in the rate-limiting step, and/or overall gene expression in the biosynthetic pathway (Sato et al., 2001, 2007; Glenn et al., 2012). Transcription factors should be powerful tools for controlling overall gene expression in biosynthesis, even though the comprehensive activator in the pathway is comparatively rare and transcription activity is generally fine-tuned. Here, we discuss the potential and current limitations of the use of transcription factors for improving the production yield of secondary metabolites.

Transcription factors are required to improve the yield due to the limitation of the traditional approach that involves overexpression of the gene for the rate-limiting step. The overexpression of a single gene is often insufficient because the metabolic flow is regulated at multiple steps. Furthermore, it is not easy to identify the rate-limiting steps in a biosynthetic pathway. Thus, a master transcription activator is required to overexpress all of the enzymes in the pathway to improve the biosynthetic flux and increase the yield. Ectopic overexpression of *Lc* and *C1*, which are bHLH and MYB transcription factors in anthocyanin biosynthesis in maize, increased flavonols in tomato fruits to 130 µg/g fresh weight, whereas no anthocyanin accumulation was observed (Bovy et al., 2002). On the other hand, the expression of *Del* and *Ros1*, which are two transcription factors in anthocyanin biosynthesis in snapdragon, resulted in higher levels of anthocyanins ( $2.83 \pm 0.46$  mg of anthocyanin per g fresh weight) in tomato (Butelli et al., 2008). These authors speculated that snapdragon transcription factors (i.e., *Del* and *Ros1*) might regulate the expression of a wider range of genes in phenylpropanoid biosynthesis than those in maize (i.e., *Lc* and *C1*), and the high induction of *CHI* by *Del* and *Ros1* might overcome the limitation of the flux of the biosynthetic pathway.

The application of transcription factors in alkaloid biosynthesis is more limited compared to that in phenylpropanoid biosynthesis due to a lack of information, especially with regard to the general function of transcription factors in other types of alkaloids. Thus, the overexpression of transcription factor(s) is generally limited to within the native host and the results are modest, as mentioned above. For example, the overexpression of *CrWRKY1* increased only the serpentine content 2.5-fold (Suttipanta et al., 2011). The transient overexpression of *CrMPK3* resulted in a 3.5-fold

increase in serpentine and a 2.5-fold increase in vindoline (Raina et al., 2012). The expression of MYC2-type transcription factors in tobacco or *C. roseus* also had only a modest effect on the expression of biosynthetic genes and alkaloid production (Zhang et al., 2011, 2012). Wang et al. (2010) found that the combined overexpression of a transcription factor, *ORCA3*, and the *ORCA3*-uncontrolled geraniol 10-hydroxylase (*G10H*) gene might be needed to increase alkaloid production when the pathway is regulated by multiple transcription regulators, whereas production of the desired dimeric vinblastine and vincristine was still low. These observations indicate that further investigations are needed on the regulatory network of alkaloid biosynthesis, and especially on the interaction of multiple transcriptional regulators.

Intriguingly, Apuya et al. (2008) demonstrated that the heterologous expression of transcription factors of a non-alkaloid-producing plant resulted in the higher production of some alkaloids. They screened transcription factors of *A. thaliana*, soybean and maize which activates the gene promoters of enzymes in alkaloid biosynthesis in *A. thaliana* and tobacco, and identified *AtWRKY1*. The overexpression of *AtWRKY1* in transgenic *E. californica* cells increased the expression of *EcCYP80B1* and *EcBBE* genes and the accumulation of dihydrosanguinarine and 10-hydroxydihydrosanguinarine up to 30-fold and 34-fold, respectively. Furthermore, the overexpression of *AtWRKY1* in *P. somniferum* plants increased the transcript levels of *PsTYDC*, *Ps6OMT* and *Ps4'OMT2* genes and increased thebaine levels 5-fold. Ectopic expression of heterologous AP2/ERF and MYB in non-alkaloid-producing plants also had similar effects on alkaloid production, suggesting that the regulatory network in alkaloid biosynthesis might be similar to those in non-alkaloid-producing plant species and information on the biosynthesis of other secondary metabolites might be useful for improving alkaloid productivity in the future.

## 6. Conclusion

Transcription factors are central regulators that control gene expression, development and responses to environmental stimuli. With the accumulation of molecular information regarding secondary metabolism, we can begin to clarify the transcriptional regulation of alkaloid biosynthesis. Several transcription factors of different types, such as AP2/ERF, WRKY and bHLH transcription factors, or regulatory factors, such as MAP kinases, have been identified in the biosynthesis of each alkaloid. Whereas these factors positively and negatively regulate gene expression and fine-tune biosynthesis, a comparison with the transcription factors involved in phenylpropanoid biosynthesis revealed similarities and differences among secondary metabolites. Rapid progress in the acquisition of genome information with the development of next-generation sequencing technology and combinatorial genetic, molecular and biochemical analysis may contribute to a full understanding of the uncharacterized metabolic pathways in divergent medicinal plants and complicated regulatory mechanisms in secondary metabolite biosynthesis. An understanding of the regulatory mechanisms in alkaloid biosynthesis in comparison to those of phenylpropanoid and terpenoid biosynthesis should be very helpful for elucidating the evolution of secondary metabolism and the development of systems for the industrial production of many important secondary metabolites in plants.

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Figure 1

MIA biosynthetic pathway. AS, anthranilate synthase; CPR, cytochrome P450 reductase; DAT, deacetylvindoline-4-*O*-acetyltransferase; D4H, desacetoxyvindoline 4-hydroxylase; G10H, geraniol 10-hydroxylase; DXS, D-1-deoxyxylulose 5-phosphate synthase; LAMT, loganic acid-*O*-methyltransferase; NMT, 16-methoxy-2,3-dihydro-3-hydroxytabersonine-*N*-methyltransferase; 16OMT, 16-hydroxytabersonine-16-*O*-methyltransferase; SLS, secologanin synthase; SGD, strictosidine *O*- $\beta$  glucosidase; STR, strictosidine synthase; TDC, tryptophan decarboxylase; T16H, tabersonine 16-hydroxylase;. Broken lines indicate more than one reaction, or uncharacterized reactions.

## Figure 2

The biosynthetic pathways for nicotine and other alkaloids. ODC, ornithine decarboxylase; PMT, putrescine *N*-methyltransferase; QPT, quinolinate phosphoribosyltransferase; MPO, *N*-methylputrescine oxidase; AOX, aspartate oxidase; QS, quinolinic acid synthase. Broken lines show more than one reaction, or uncharacterized metabolic pathways.

## Figure 3

BIA biosynthetic pathway. The metabolic pathway from L-tyrosine to (*S*)-reticuline is the common pathway in *C. japonica*, *E. californica* and *P. somniferum*. *C. japonica* exhibits the pathways from (*S*)-scoulerine to berberine and from (*S*)-reticuline to magnoflorine. *E. californica* mainly produces sanguinarine from (*S*)-scoulerine. *P. somniferum* exhibits the pathway from (*S*)-reticuline to morphine through (*R*)-reticuline. TYDC, tyrosine decarboxylase; NCS, norcoclaurine synthase; 6OMT, norcoclaurine 6-*O*-methyltransferase; CNMT, coclaurine-*N*-methyltransferase; CYP80B1, *N*-methylcoclaurine 3'-hydroxylase; 4'OMT, 3'-hydroxy-*N*-methylcoclaurine-4'-*O*-methyltransferase; BBE, berberine bridge enzyme; SMT, scoulerine-9-*O*-methyltransferase; CYP719A1, canadine synthase; THBO, tetrahydroberberine oxidase; CYP80G2, corytuberine synthase; CYP719A5, cheilanthifoline synthase; CYP719A2/3, stylophine synthase; TNMT, tetrahydroberberine *cis-N*-methyltransferase; MSH, *N*-methylstrylophine 14-hydroxylase; P6H, protopine-6-hydroxylase; DBOX, dihydrobenzophenanthridine oxidase; CYP719B1, salutaridine synthase; SalR, salutaridine 7-oxidoreductase; SalAT, salutaridinol-7-*O*-acetyltransferase; T6ODM, thebaine 6-*O*-demethylase; CODM, codeine *O*-demethylase; COR, codeinone reductase. Broken lines indicate more than one reaction, or uncharacterized reactions.

#### Figure 4

Simplified transcriptional network of JA signaling in *A. thaliana* (A), nicotine biosynthesis in *N. tabacum* and *N. benthamiana* (B), MIA biosynthesis in *C. roseus* (C) and BIA biosynthesis in *C. japonica* (D). Broken lines indicate unidentified proteins or regulation. Arrows indicate up-regulation and T-shaped lines indicate inhibition.

#### Figure 5

Simplified model of the JA-mediated signaling cascade in anthocyanin, sesquiterpene and alkaloid biosynthesis. Arrows indicate up-regulation and T-shaped lines indicate inhibition.



Table 1 Transcription factors that have been identified in alkaloid biosynthesis

Family	Name	Accession	Plant species
AP2/ERF	ORCA2	AJ238740	<i>Catharanthus roseus</i>
	ORCA3	EU072424	<i>C. roseus</i>
	ERF189	AB827951	<i>Nicotiana tabacum</i>
	ERF221/ORC1	CQ808982	<i>N. tabacum</i>
WRKY	CjWRKY1	AB267401	<i>Coptis japonica</i>
	CrWRKY1	HQ646368	<i>C. roseus</i>
bHLH	NbbHLH1	GQ859152	<i>Nicotiana benthamiana</i>
	NbbHLH2	GQ859153	<i>N. benthamiana</i>
	NtMYC2a	HM466974	<i>N. tabacum</i>
	NtMYC2b	HM466975	<i>N. tabacum</i>
	CrMYC2	AF283507	<i>C. roseus</i>
	CjbHLH1	AB564544	<i>C. japonica</i>
MYB-like	BPF-1	AJ251686	<i>C. roseus</i>
bZIP	GBF1	AF084971	<i>C. roseus</i>
	GBF2	AF084972	<i>C. roseus</i>
TFIIIA zinc finger	ZCT1	AJ632082	<i>C. roseus</i>
	ZCT2	AJ632083	<i>C. roseus</i>
	ZCT3	AJ632084	<i>C. roseus</i>
AT-hook	2D328	EF025306	<i>C. roseus</i>
	2D173	EF025307	<i>C. roseus</i>
	2D449	EF025308	<i>C. roseus</i>
	2D38M	EF025309	<i>C. roseus</i>
	2D7	EF025310	<i>C. roseus</i>

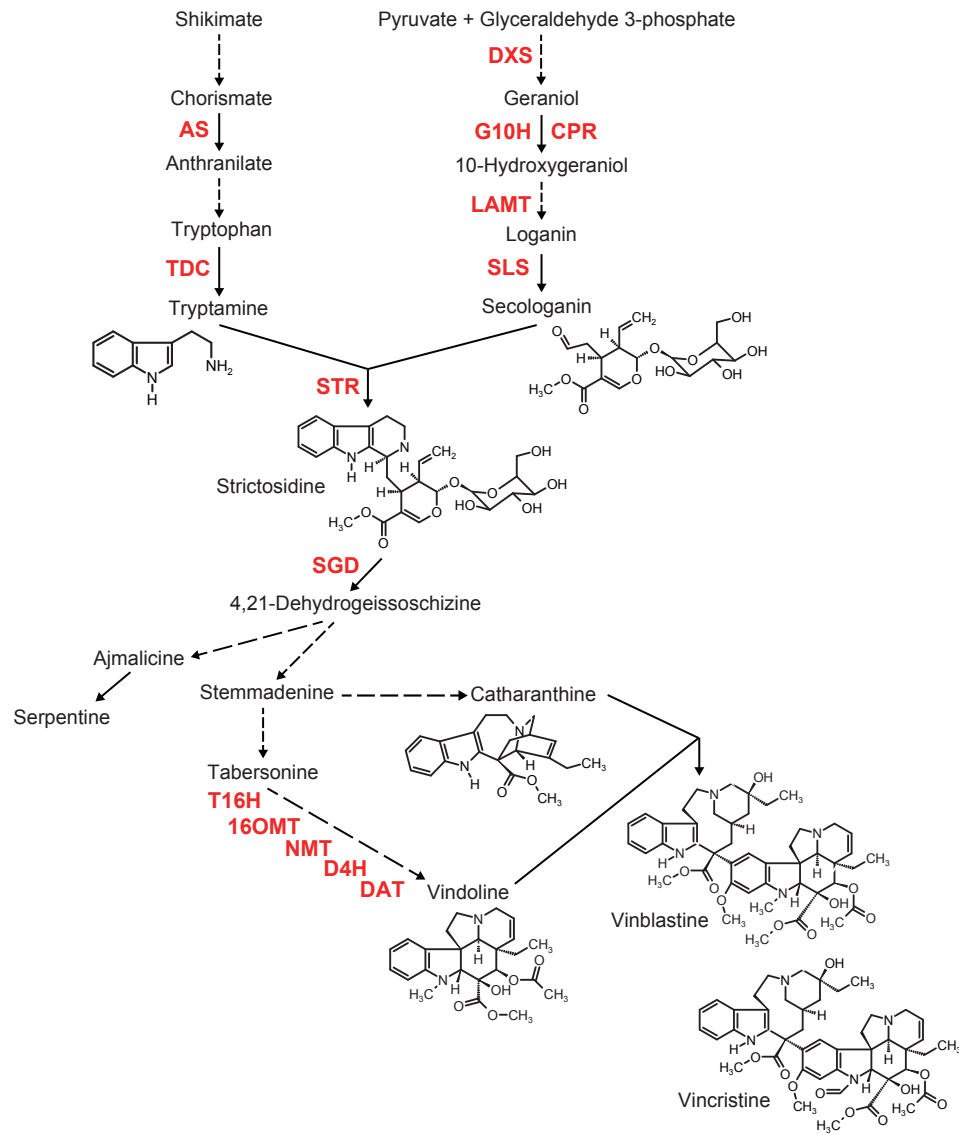


Fig. 1

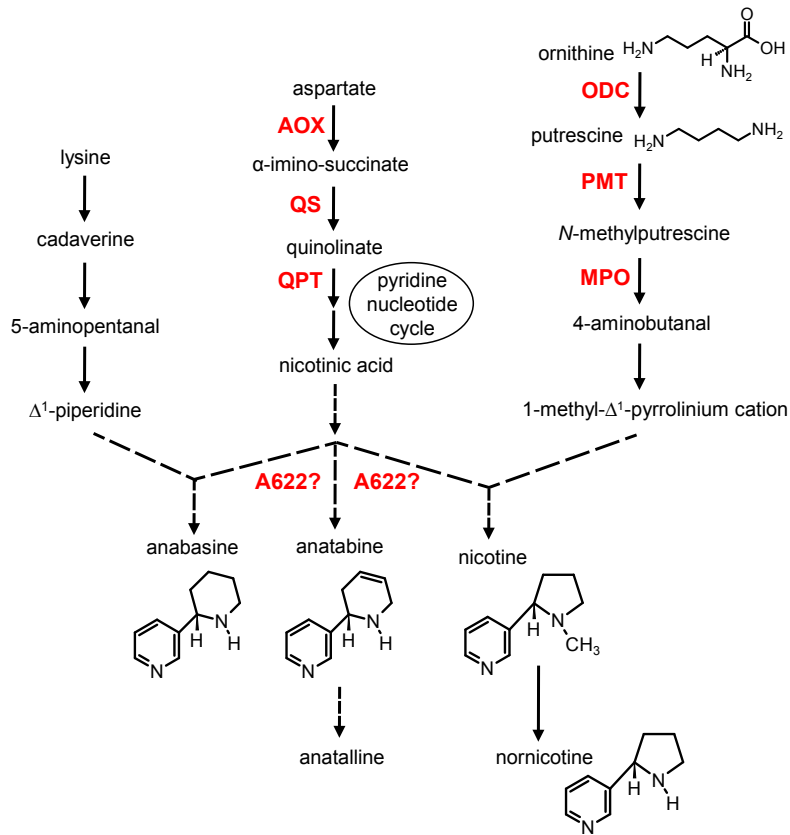


Fig. 2

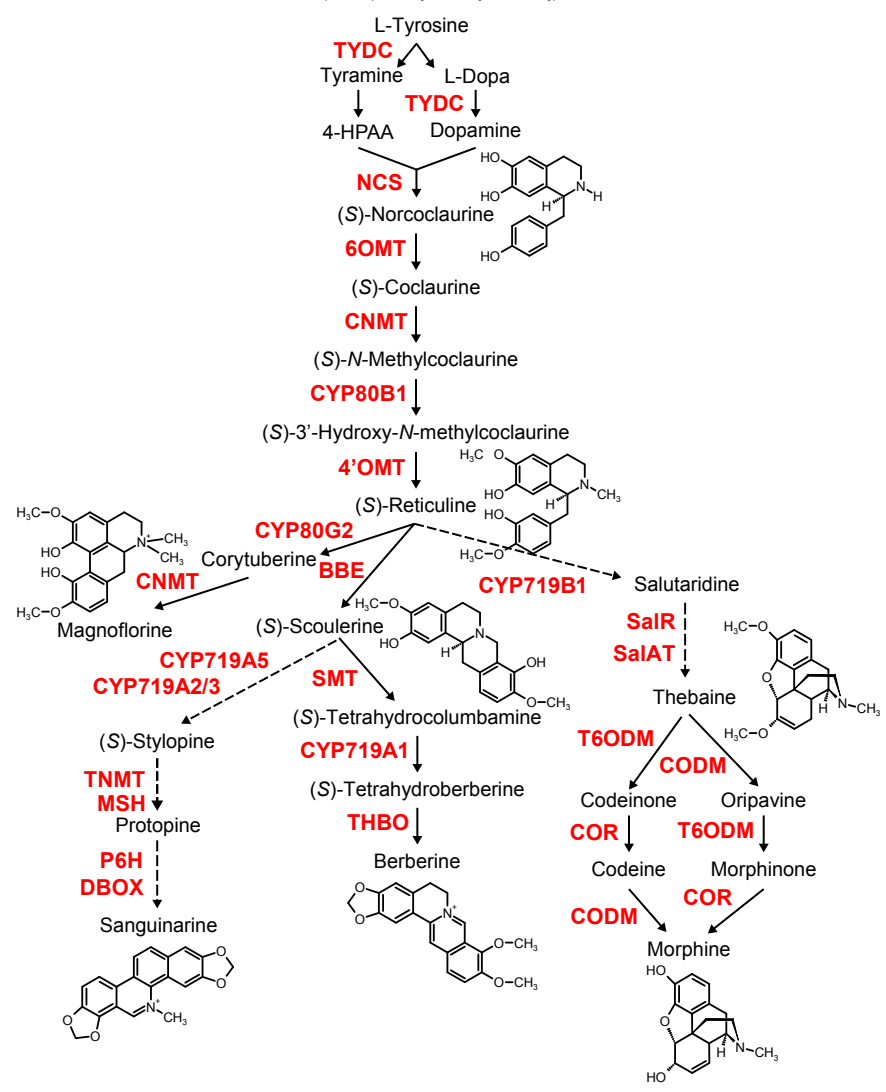
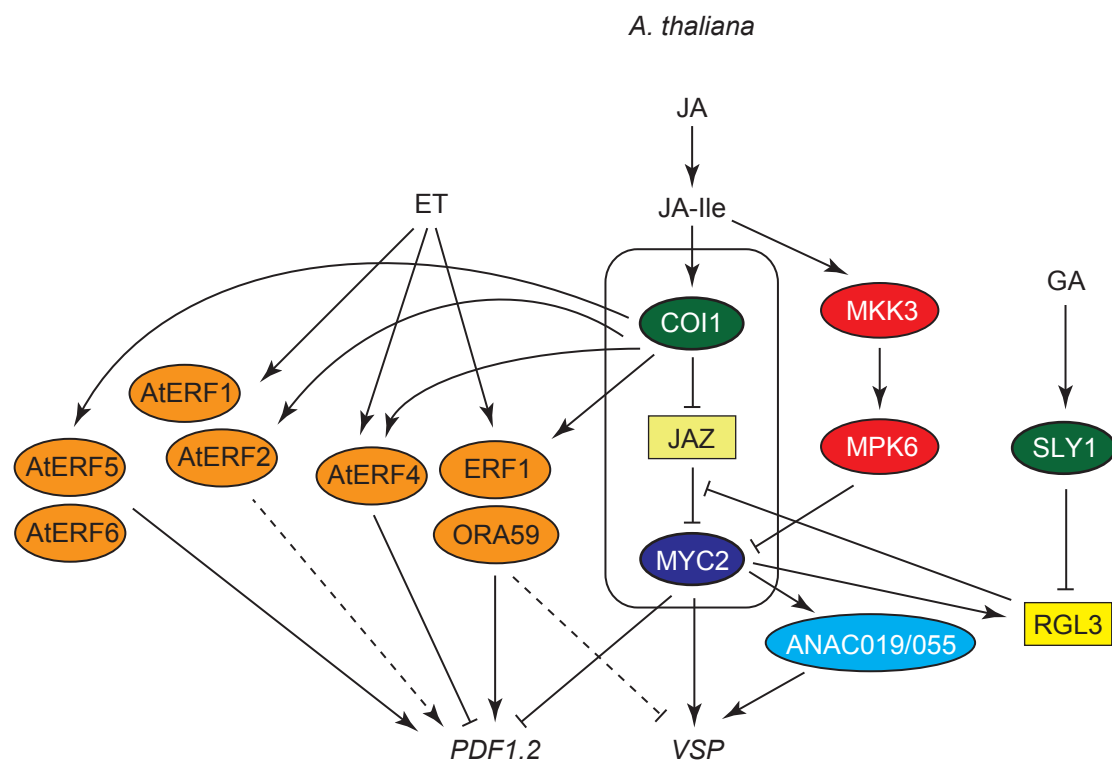
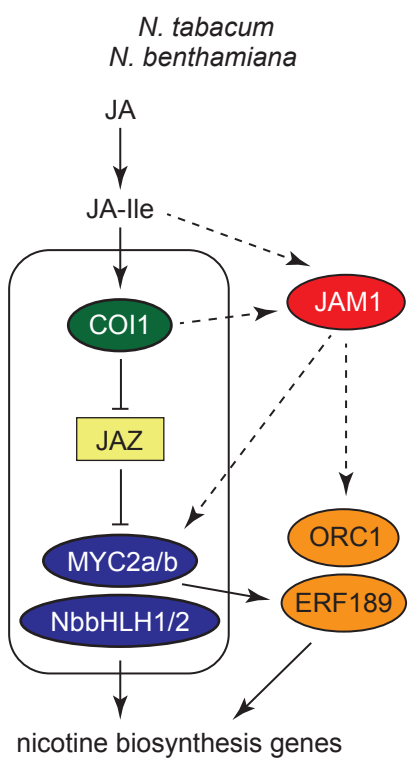


Fig. 3

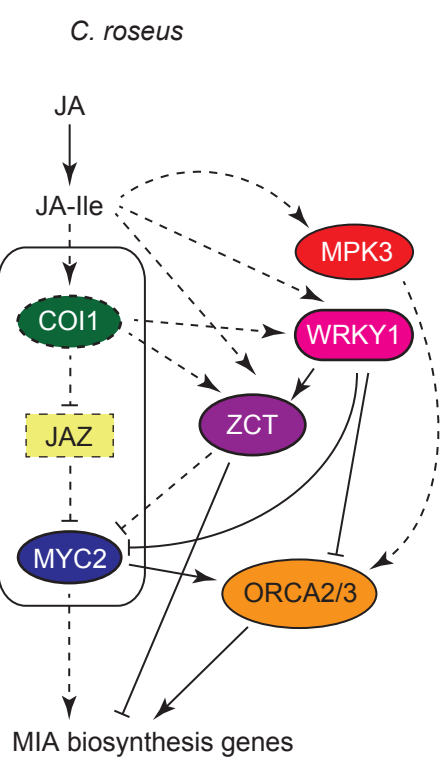
A



B



C



D

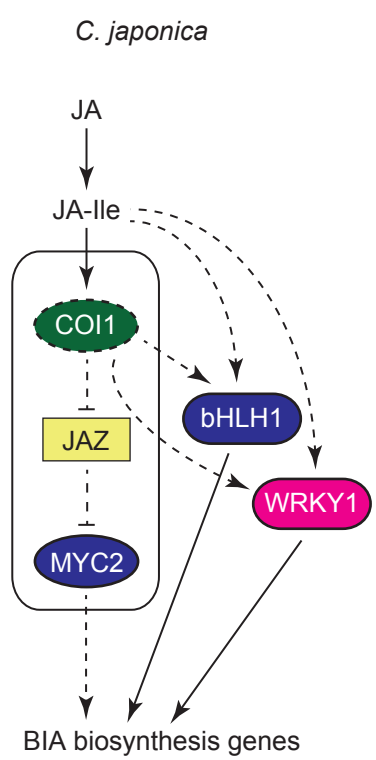


Fig. 4



Fig. 5